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The potential of cloning depends in part on whether the procedure can reverse cellular aging and restore somatic cells to a phenotypically youthful state. Here, we report the birth of six healthy cloned calves derived from populations of senescent donor somatic cells. Nuclear transfer extended the replicative life-span of senescent cells (zero to four population doublings remaining) to greater than 90 population doublings. Early population doubling level complementary DNA-1 (EPC-1, an age-dependent gene) expression in cells from the cloned animals was 3.5- to 5-fold higher than that in cells from age-matched (5 to 10 months old) controls. Southern blot and flow cytometric analyses indicated that the telomeres were also extended beyond those of newborn (<2 weeks old) and age-matched control animals. The ability to regenerate animals and cells may have important implications for medicine and the study of mammalian aging.

Questions have been raised as to whether cells or organisms created by nuclear transfer will undergo premature senescence. Normal somatic cells display a finite replicative capacity when cultured in vitro (1, 2). The germ line appears to maintain an immortal phenotype in part through expression of the ribonucleoprotein complex telomerase, which maintains the telomeres at a long length. However, nuclear transfer technologies use embryonic, fetal, and adult somatic cells that often do not express telomerase from a range of mammalian species (3-10). A recent report (11) suggests that nuclear transfer may not restore telomeric length and that the terminal restriction fragment size observed in animals cloned from cells reflects

the mortality of the transferred nucleus, which could limit the utility of the cloning of replacement cells and tissue for human transplantation (12, 13).

Wilmut *et al.* (3) have reported that arrest in the G₀ phase of the cell cycle is required to obtain normal development of animals cloned from differentiated cells. Replicative senescence is a physiological state distinguishable from quiescence achieved by either serum starvation or density-dependent inhibition of growth of young cells (14-18) and appears to involve a block in late G₁ near the G₁/S boundary in the cell cycle (19-21), possibly reflecting a DNA checkpoint arrest (22-26). Here we investigate whether the production of live offspring is possible by nuclear transfer of late-passage somatic cells and whether the epigenetic changes seen in the donor cells, such as telomere shortening and loss of replicative life-span, are reflected in the resultant organism.

A somatic cell strain was derived from a 45-day-old female bovine fetus (BFF) and transfected with a PGK-driven selection cassette. Cells were selected with G418 for 10 days, and five neomycin-resistant colonies were isolated and analyzed for stable trans-

fection by Southern blotting with a full-length cDNA probe. One cell strain (CL53) was identified as 63% (total nuclei) positive for the transgene by fluorescence in situ hybridization (FISH) analysis and was chosen for our nuclear transfer studies. These fibroblast cells, which were negative for cytokeratin and positive for vimentin, were passaged until greater than 95% of their life-span was completed, and their morphology was consistent with cells close to the end of their life-span (Fig. 1A).

A more detailed ultrastructural analysis by electron microscopy demonstrated that these cells exhibited additional features of replicative senescence, including prominent and active Golgi apparatus, increased invaginated and lobed nuclei, large lysosomal bodies, and an increase in cytoplasmic microfilaments as compared with the young cells (Fig. 1B) (27). In addition, these late-passage cells exhibited a senescent phenotype in showing a reduced capacity to enter S phase (Fig. 1C) and a significant increase in the staining of senescence-associated β -galactosidase (28, 29). Furthermore, these cells exhibited a reduction in EPC-1 (early population doubling level cDNA-1) (30) mRNA levels as compared with early-passage bovine BFF cells in a manner analogous to the changes observed during the aging of WI-38 cells (Fig. 1D).

A total of 1896 bovine oocytes were reconstructed by nuclear transfer with senescent CL53 cells (4). Eighty-seven blastocysts (5%) were identified after a week in culture. The majority of the embryos ($n = 79$) were transferred into progestin (SYNCRIMATE-B)-synchronized recipients (2 to 6 years old), and 17 of the 32 recipients (53%) were pregnant by ultrasound 40 days after transfer. One fetus was electively removed at week 7 of gestation (ACT99-002), whereas nine (29%) remained pregnant by 12 weeks of gestation. Two of these aborted at days 252 (twins) and 253, and one was delivered stillborn at day 278. The remaining six recipients continued development to term. The rates of blastocyst formation (5%) and early (53%) and term (19%) pregnancies with senescent CL53 cells were comparable to those of control embryos produced with nonsenescent donor (CL57) cells from early-passage cells (5, 45, and 13%, respectively).

Six calves were delivered by elective cesarean section (Fig. 2). Genomic analyses confirmed the presence of the transgene in two of the animals (CL53-1 and CL53-12), as well as in the fetus that was removed electively at day

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49. At birth, the presentation of the cloned calves was consistent with previous published reports (4, 6, 31, 32). In general, birth weights (51.6 ± 3.6 kg) were increased, and several of the calves experienced pulmonary hypertension and respiratory distress at birth, as well as incidence of fever after vaccinations at 4 months. After the first 24 hours, the calves were vigorous with minimal health problems. However,

we noted a moderate incidence of polyuria/polydipsia and lowered dry matter intake during the first two months. The occurrence of these complications was linked neither to the donor cell population (isolate 53 or 57) nor to the presence or absence of transgene integration. After about 2 months, all of the calves resembled healthy control calves generated from both in vitro fertilization and in vivo

zygo transfers, and they remained alive and normal 7 to 12 months after birth. Messenger RNA from dermal fibroblasts of the cloned calves was isolated (Fig. 1D). The cells from the cloned animals expressed about threefold higher EPC-1 mRNA levels than the early-passage fetal bovine cells. Furthermore, these dermal fibroblasts also expressed a 3.5- to 5-fold higher level of EPC-1 mRNA than comparable lines derived from age-matched control animals. This suggests that the fibroblasts derived from the cloned animals are potentially younger than the control fibroblasts.

To confirm that results from the cloned calves were not due to variations in the donor cell population, we produced dermal fibroblasts from three adult Holstein steers. Single-cell clones were isolated, and population doublings were counted until senescence. Nuclear transfer was performed with those fibroblast cells that were at or near senescence. Fetuses were removed from the uterus at week 6 of gestation, and fibroblasts were isolated from them and cultured until senescence. Cells were analyzed by immunohistochemistry and were shown to be fibroblasts. Cell strains isolated from the cloned fetuses underwent an average of 92.6 ± 1.6 population doublings as compared with 60.5 ± 1.7 population doublings for cell strains generated from normal age-matched (6-week-old) control fetuses (Table 1) ($P < 0.0001$). These data suggest that cloning is capable of resetting (indeed, extending) the life-span of somatic cells and that the cellular age of the fetus does not reflect the number of times the donor cells doubled in culture before nuclear transfer.

To exclude the possibility that there was a small proportion of nonsenescent cells that gave rise to the cloned animals, we seeded CL53 donor cells at both normal and clonal densities. The cells were 2.01 ± 0.11 (SEM) population doublings from replicative senescence. Less than 12% (11/97) and 3% (2/97) of cells seeded at clonal density underwent more than one or

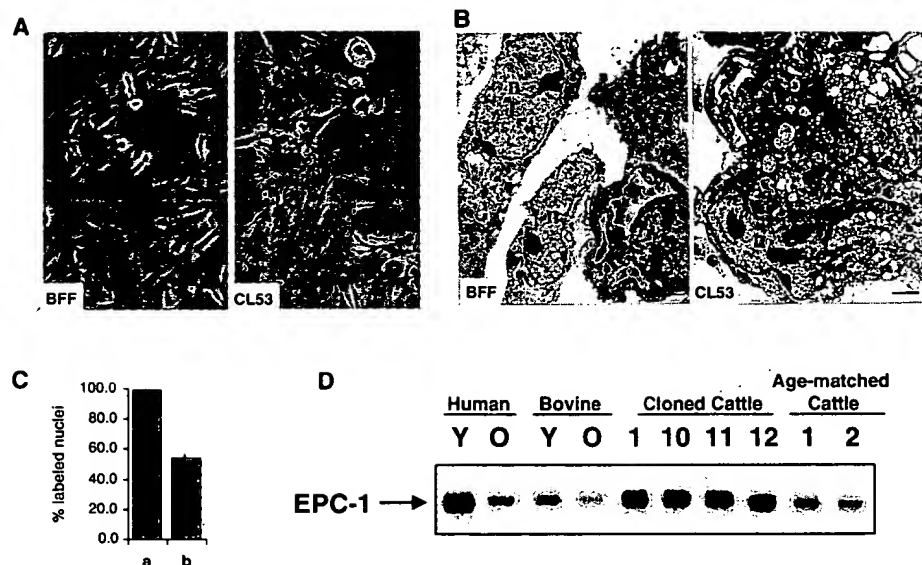


Fig. 1. Characterization of cell senescence in nuclear transfer donor cells. (A) Cells were observed by phase contrast microscopy. The donor cells (CL53) displayed an increased cell size and cytoplasmic granularity as compared with the early-passage BFF cells. (B) Representative electron micrographs of BFF and donor CL53 cells. Note the convoluted nucleus (n) of CL53 cells. CL53 cells are larger than BFF cells, and their cytoplasm contains abundant lysosomes (arrows) and thick fibrils. Both pictures are at the same magnification. Bar, 2 μ m. Mitochondria (m). (C) Entry of early- (BFF, a) and late-passage (CL53, b) cells into DNA synthesis as determined by 3 H-thymidine incorporation during a 30-hour incubation (40). The cells were processed for autoradiography and then observed microscopically and scored for labeled nuclei. At least 400 nuclei were counted (40). (D) The donor CL53 cells exhibit reduced EPC-1 mRNA levels as determined by Northern blot analysis. Human fibroblasts (WI-38) at early passage (Y) and late passage (O), bovine fibroblasts at early passage (Y; BFF) and late passage (O; donor CL53), RNAs isolated from cloned cattle (animals CL53-1, CL53-10, CL53-11, and CL53-12), and age-matched control (animals 1 and 2) dermal fibroblast strains are indicated. Total RNA was extracted from the cells after they were grown to confluence and growth-arrested in serum-free medium for 3 days (41). Equal amounts of RNA were treated with glyoxal, separated by electrophoresis on agarose gels, electrophoretically transferred to positively charged nylon membranes, and hybridized with the full-length EPC-1 cDNA (42).

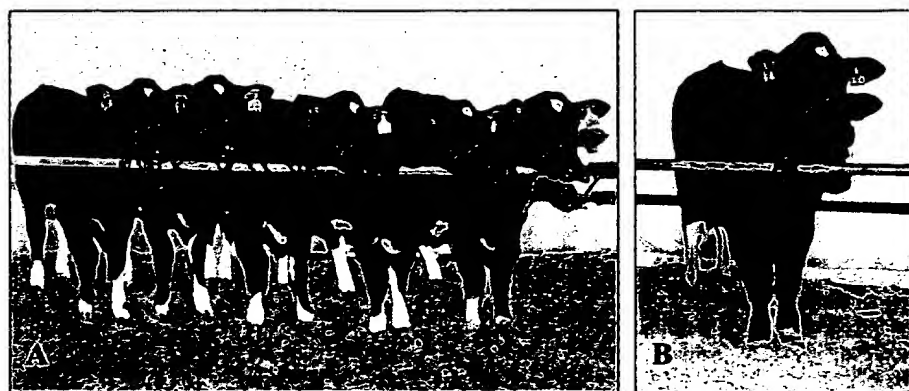


Fig. 2. Normal heifers cloned from senescent somatic cells. (A) CL53-8, CL53-9, CL53-10, CL53-11, and CL53-12 (nicknamed Lily, Daffodil, Crocus, Forsythia, and Rose, respectively) at 5 months of age and (B) CL53-1 (Persephone) at 10 months of age.

Table 1. Population doublings in fibroblasts derived from normal fetuses and fetuses generated from clonal populations of adult senescent cells.

	PDs left at time of nuclear transfer in original adult cells	PDs in fibroblasts isolated from the fetus
<i>Cloned fetus</i>		
25-1	0.26	90.1
25-2	0.0	91.4
14-1	4.0	89.3
14-2	1.0	99.2
22-1	2.5	92.9
<i>Normal fetus</i>		
1-1	—	59.6
2-1	—	67.4
3-1	—	60.2
3-2	—	59.8
3-3	—	55.7

two population doublings, respectively, while as none of the cells divided more than three times (Fig. 3C). These data are consistent with a second experiment that was performed in which 250 cells were seeded at clonal densities (none of the cells underwent more than four population doublings). In contrast, early-passage (pretransfection) BFF cells underwent 58.7 ± 1.2 population doublings, with an average cell cycle length of 17.8 ± 0.7 hours during the logarithmic growth phase (Fig. 3A).

To test whether the somatic cell nuclear transfer procedure restored the proliferative life-span of the senescent donor fetal cells, we cultured fibroblasts from an electively removed 7-week-old fetus (ACT99-002). Cell strains from it underwent 96.1 ± 7.3 population doublings, with a cell cycle length of 17.7 ± 0.8 hours during the logarithmic growth phase (Fig. 3A). One-cell clones ($n = 5$) were generated from the cloned (ACT99-002) and original (BFF) age-matched fetuses, and cultures characterized as fibroblasts by immunohistochemical staining were isolated. These one-cell clones underwent 31.2 ± 3.4 and 25.9 ± 2.9 population doublings from the cloned and original fetuses, respectively (Fig. 3D).

To further investigate the ability of nuclear transfer to rescue senescent cells, we compared the telomere lengths in nucleated blood cells of the six cloned animals with those of age-matched (5 to 10 months old) control animals, newborn calves (<2 weeks old), and cows of various ages (ranging from 6 months to 19 years old) using flow cytometric analysis after *in situ* hybridization with directly fluorescein isothiocyanate (FITC)-labeled (CCCTAA) peptide nucleic acid probe (flow FISH) (Fig. 4, A and B) (33, 34). The results of three separate experiments are indicative of elongation of telomere length in the cloned animals relative to age-matched controls [63.1 ± 1.7 compared with 50.8 ± 2.9 kMESF (molecules of equivalent soluble fluorochrome) (mean \pm SD, $P < 0.0001$, experiment 1), 75.4 ± 1.5 compared with 60.8 ± 3.1 kMESF ($P < 0.0001$, experiment 2), and 73.6 ± 0.3 compared with 62.7 ± 4.0 kMESF ($P < 0.0001$, experiment 3)]. Indeed, in two of three experiments, the telomeres in cells of the cloned animals were significantly longer than those in cells from the newborn calves [75.4 ± 1.5 compared with 66.8 ± 5.1 kMESF ($P < 0.0002$, experiment 2) and 73.6 ± 0.3 compared with 62.7 ± 4.0 kMESF ($P < 0.0001$, experiment 3)]. The mean telomere lengths in nucleated bovine blood cells showed considerable variation at any given age as in human nucleated blood cells (34). Nevertheless, a highly significant decline in telomere length with age was observed ($P < 0.001$), corresponding to an estimated 20 to 100 base pairs of telomere repeats per year ($n = 46$). More extensive studies are needed to establish the rate of telomere shortening in the various nucleated blood cells from cattle.

Telomere length was also studied with Southern blot analysis of terminal restriction fragments (22). The results (Fig. 4C) obtained with senescent (CL53), control (pretransfection BFF), and cloned (ACT99-002) cells were consistent with the flow FISH analysis of nucleated blood cells. The telomeres were longer in the cells derived from the cloned fetus (20.1 kb, lanes 3 and 6) than in the senescent and early-passage donor cells (15.2 and 18.3 kb, respectively; compare lanes 1 to 6, Fig. 4C). These results were reproduced in two separate experiments and were consistent with flow FISH analysis on the same cells (28).

The telomere length in clonal populations of senescent adult dermal fibroblasts (0.26 to 2.5 population doublings remaining) was compared with that in fibroblasts from cloned fetuses obtained with these cells (Fig. 4C, lanes 7 to 10). In the two cases that could be analyzed, an increase in telomere length was also observed upon cloning from senescent fibroblasts. The increase in telomere length ranged from 14.4 to 16.4 kb for clone 22-1 to from 12.1 to 16.1 kb for clone 25-1. The telomere length in these cloned early-passage (<10 population doublings) fibroblasts with extensive proliferative potential (Table 1) was comparable to that of the senescent fi-

broblasts that gave rise to the cloned animals with elongated telomeres described in this report. These results highlight the variable terminal restriction fragment length associated with replicative senescence.

High levels of telomerase activity were detected in reconstructed day 7 embryos tested by the telomeric repeat amplification protocol (TRAP) assay (Fig. 5, lanes 5 to 8), whereas the bovine fibroblasts used as donor cells in the nuclear transfer experiments were negative (Fig. 5, lane 9).

Our results differ from the study by Shiels *et al.* (11), in which telomere erosion did not appear to be repaired after nuclear transfer in sheep. The telomere lengths in cells from three cloned animals, 6LL3 (Dolly, obtained from an adult donor cell), 6LL6 (derived from an embryonic donor cell), and 6LL7 (derived from a fetal donor cell), were decreased relative to those of age-matched control animals. The authors suggested that full restoration of telomere length did not occur because these animals were generated without germ line involvement. They further suggested that the shorter terminal restriction fragment in Dolly was consistent with the time the donor cells spent in culture before nuclear transfer. Our findings show that viable offspring can be produced from senescent

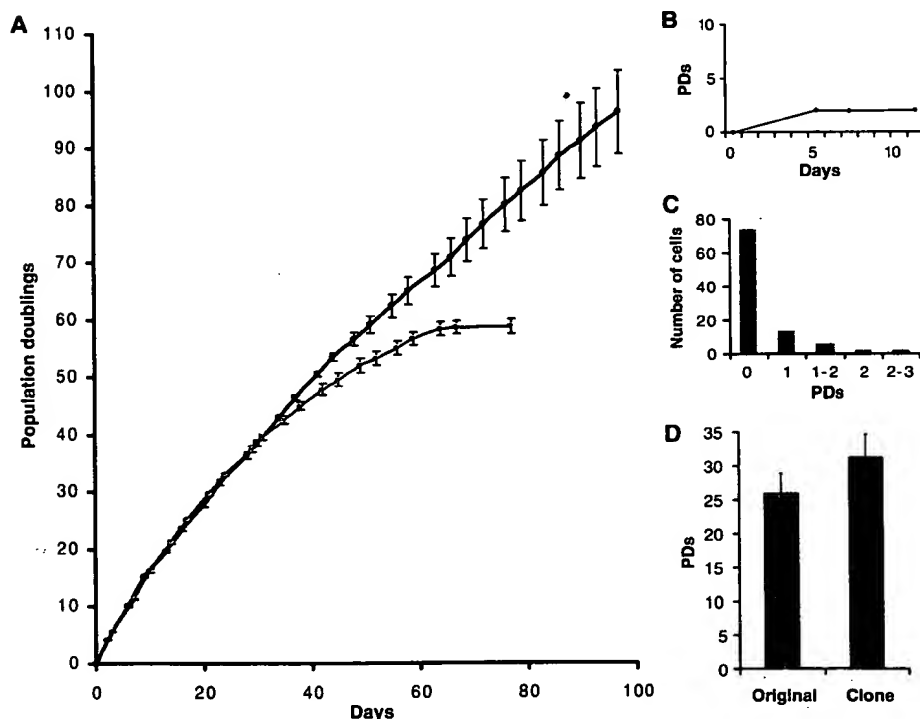


Fig. 3. Ability of nuclear transfer to restore the proliferative life-span of senescent donor cells. (A) The growth curve of the original BFF cell strain (green) is compared with that of cells derived from the fetus (ACT99-002) (black) that was cloned from late-passage BFF cells (CL53 cells). (B) The growth curve of the CL53 donor cells demonstrating that the cultures had about two population doublings (PDs) remaining. (C) Late-passage CL53 cells ($n = 97$) were seeded at clonal density, and the proliferative capacity after 1 month was determined. (D) Single-cell clones from early-passage BFF cultures (original) and early-passage ACT99-002 (clone) showed a capacity for extended proliferation.

somatic cells and that nuclear transfer could extend the telomeres of the animals beyond that of newborn and age-matched control animals. It is not known whether the longevity of these animals will be reflected by telomeric measurements, although cells derived from cloned fetuses had an about 50% longer proliferative life-span than those obtained

from same-age nonmanipulated fetuses. The ability to extend the life-span of specific differentiated cell types, such as hepatocytes, cardiomyocytes, and islets, an extra 30 population doublings would lead to a billionfold increase in the number of replacement cells generated for tissue engineering and transplantation therapies.

The differences between the present study and that reported by Shiels *et al.* (11) could be due to species differences and/or differences in nuclear transfer techniques or donor cell types. Wilmut *et al.* (3), for instance, used quiescent mammary cells to produce Dolly, whereas senescent fibroblasts were used in the present experiments. Also, although recent studies have shown that reconstruction of telomerase activity can lead to telomere elongation and immortalization of human fibroblasts (35, 36), similar experiments with mammary epithelial cells did not result in elongation of telomeres and extended replicative life-span (37). Differences between cells in telomere binding proteins (38), the ability of telomerase to extend telomeres, or differences in the signaling pathways activated upon adaptation to culture (39) could explain the differences. The elongation of telomeres in the present study suggests that reconstructed bovine embryos contain a mechanism for telomere length regeneration, providing chromosomal stability throughout the events of pre- and postattachment development. The ability of nuclear transfer to restore somatic cells to a phenotypically youthful state may have important implications for agriculture and medicine.

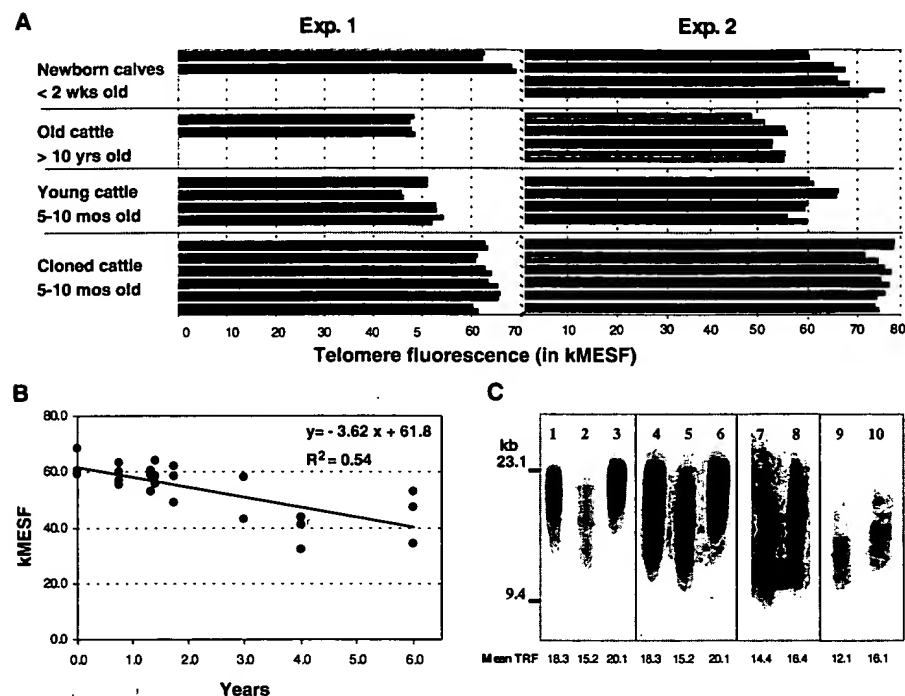


Fig. 4. Telomere length analysis. (A) Nucleated blood cells. Peripheral blood samples from cloned and control Holsteins were analyzed by flow FISH (34) in two separate blinded experiments. Duplicate samples (red and blue bars) of nucleated cells obtained after osmotic lysis of red cells with ammonium chloride were analyzed by flow FISH as described (33). The average telomere fluorescence of gated single cells was calculated by subtracting the mean background fluorescence from the mean fluorescence obtained with the FITC-labeled telomere probe. (B) Telomere lengths in nucleated blood cells of 25 normal Holsteins ranging from <2 weeks to 6 years of age, showing the decline in mean telomere lengths against age. (C) Elongation of telomeres in cells upon nuclear transfer. Terminal restriction fragment (TRF) analysis of DNA fragments obtained after digestion with Hinf I-Rsa I was performed on a 0.5% agarose gel run for 28 hours, as described (Telomere Length Assay Kit; Pharmingen, San Diego, California). Lanes 1 and 4, genomic DNA isolated from control cells (pretransfection BFF bovine fibroblasts) (mean TRF length = 18.3 kb); lanes 2 and 5, senescent CL53 cells (mean TRF length = 15.2 kb); lanes 3 and 6, fibroblasts from a 7-week-old cloned fetus (ACT99-002) obtained by nuclear transfer with senescent CL53 cells (mean TRF length = 20.1 kb) (lanes 4 to 6 are longer exposures of lanes 1 to 3); lane 7, senescent donor fibroblast clone 22-1 (mean TRF length = 14.4 kb); lane 8, nuclear transfer fetal fibroblasts obtained with senescent 22-1 cells (mean TRF length = 16.4 kb); lane 9, senescent fibroblast clone 25-1 (mean TRF length = 12.1 kb); and lane 10, nuclear transfer fetal fibroblasts obtained with senescent 25-1 cells (mean TRF length = 16.1 kb).



Fig. 5. Telomerase is expressed in reconstructed embryos but not in donor bovine fibroblasts. Telomerase activity was measured with a TRAP assay kit (Pharmingen, San Diego, California). Lysates from adult donor senescent (CL53) fibroblasts and day 7 reconstructed bovine embryos ($n = 15$) were obtained and used in the TRAP assay. Lane 1, extract from 4000 K562 human erythroleukemia cell line cells; lane 2, 20-base pair ladder; lane 3, no cell extract; lane 4, heat-treated embryo ($n = 1$) extract; lane 5, embryo extract ($n = 10$); lane 6, $n = 1$; lane 7, $n = 0.1$; lane 8, $n = 0.01$; and lane 9, extract from 4000 donor CL53 fibroblasts. All lanes contain the internal control TRAP reaction (36 base pairs, arrow).

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